

The University of Chicago

A STUDY OF THE PRODUCTION OF STAPHY-
LOCOCCAL ENTEROTOXIN IN CHEMI-
CALLY DEFINED MEDIUMS

A PART OF A DISSERTATION SUBMITTED TO THE FACULTY OF THE DIVISION OF THE
BIOLOGICAL SCIENCES IN CANDIDACY FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

DEPARTMENT OF BACTERIOLOGY
AND PARASITOLOGY

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The culture mediums previously used for the production of staphylococcal enterotoxin have been complex preparations containing meat infusions or hydrolysates of natural proteins of unknown and variable composition. Since the production of a potent enterotoxin in a simple medium of known and constant composition might facilitate studies of the nature of enterotoxin, and since information regarding the nutritional requirements for enterotoxin production seem important to an understanding of the failure of certain natural foods to support enterotoxin formation, a study has been made to determine whether staphylococci can produce enterotoxin in simple mediums composed of amino acids, vitamins, inorganic salts and glucose.

The first experimental demonstration that food poisoning could be caused by staphylococci was supplied in 1914 by Barber.¹ He noted typical food poisoning symptoms following ingestion of milk inoculated with a white staphylococcus isolated from bovine mastitis. More recent methods for production of enterotoxin have, in general, been adapted from those used in studies dealing with growth of staphylococci and production of hemolysins. Dack, Cary, Woolpert and Wiggers² demon-

strated the presence of the toxic factor in filtrates of veal infusion broth cultures. Later, Woolpert and Dack³ obtained more potent toxins in semi-solid veal infusion agar cultures similar to those used by Burnet⁴ for production of hemolysin. Dolman and Wilson⁵ used a proteose-peptone semi-solid agar. In a study of various toxins formed by staphylococci, Darrach⁶ employed the latter medium and also one containing hydrolyzed edestin. Favorite and Hammon⁷ found that enterotoxin was produced in a liquid medium consisting of a 1.5% casein hydrolysate to which glucose and vitamins were added. Several workers have reported hemolysin production in hydrolyzed protein, beef-peptone dialysate and meat extract mediums.^{8,9,10,11,12} In some instances hemolysin production was increased when certain amino acids were added to the mediums.

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3. Woolpert, Oram C., and Dack, G. M. 1933, *J. Infect. Dis.* **52**: 6-19.
 4. Burnet, F. M. 1930, *J. Path. Bact.* **33**: 1-16.
 5. Dolman, C. E., and Wilson, R. J. 1938, *J. Immunol.* **35**: 13-30.
 6. Darrach, M. D. 1941, University of Toronto Ph.D. Thesis.
 7. Favorite, Grant O., and Hammon, William McD. 1941, *J. Bact.* **41**: 305-316.
 8. Knight, B. C. J. G. 1935, *Brit. J. Exper. Path.* **16**: 315-326.
 9. Holt, Lewis B. 1936, *Brit. J. Exper. Path.* **17**: 318-323.
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 11. Gengou, O. 1935, *Ann. l'Inst. Pasteur.* **55**: 129-147.
 12. Ramon, Gaston, Boivin, Andre, and Richou, Remy. 1938, *Compt. Rend. Acad. Sci.* **207**: 466-468.
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- This paper is a revision for publication of a paper submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Chicago.
- This study was aided by a grant from the National Canners Association.
1. Barber, B. M. 1914, *Philippine J. of Sci.* **9**: 515-519.
 2. Dack, G. M., Cary, W. E., Woolpert, Oram,

The vitamin requirements for growth of staphylococci were worked out by Knight^{13,14} who found that nicotinic acid and thiamine could replace the yeast and meat extracts used formerly to stimulate growth.^{8,15} Growth of certain strains was found to be stimulated by biotin¹⁶ and pantothenic acid.¹⁷

The nitrogen requirements of staphylococci vary with different strains. Fildes, Richardsdon, Knight and Gladstone¹⁸ obtained good growth in a medium consisting of 14 amino acids, inorganic salts and yeast extract. Gladstone¹⁹ found that the amino acids most necessary for rapid growth were cystine, leucine, valine, proline, glycine, aspartic acid, phenylalanine and arginine. However, most strains could be grown in the absence of any one of the more important acids with the exception of cystine. One strain was trained to grow in the absence of cystine in a medium whose only source of nitrogen was ammonia and minute quantities of thiamine and nicotinamide. Fildes and Richardson²⁰ reported that cystine could be replaced by other organic sulfur compounds containing a sulfhydryl group. All of the mercapto-compounds which they tested were active to some extent, but none were as active as cystine. Gladstone²¹

studied the production of alpha-hemolysin under controlled conditions in simple mediums modified from that of Fildes et al.¹⁸ He found that the most important amino acids were arginine, glycine, proline, phenylalanine and valine. In this study Gladstone also noted the effect of glucose and certain inorganic ions. The optimum glucose concentration was M/80; without glucose, growth and lysin production were impaired. Magnesium and iron were interchangeable to some extent; in the absence of both no lysin was formed and growth was slightly impaired. Calcium ions were inhibitory and the other inorganic salts tested had no effect.

The favorable effect of increased CO₂ tension for production of toxin by staphylococci was first noted by Parker, Hopkins and Gunther.²² These workers obtained more potent dermatotoxin when cultures were incubated in an atmosphere containing 10% CO₂. Increased CO₂ tension was also found to be beneficial for production of alpha-lysin by Burnet⁴ and of beta-lysin by Bigger.²³ Woolpert and Dack³ employed an atmosphere containing 20–25% CO₂ for production of enterotoxin.

The beneficial effect of agitation of liquid cultures for lysin production was noted by Ramon, Boivin and Richou¹² who rotated their cultures in round flasks. Casman²⁴ obtained high titers of alpha-lysin by continuous rocking of broth cultures in shallow layers. Favorite and Hammon⁷ rotated their cultures in nursing bottles for production of enterotoxin. Duthie and Wylie²⁵ found that shaking of cultures had a favorable

13. Knight, B. C. J. G. 1937, *Biochem. J.* **31**: 731–737.

14. Knight, B. C. J. G. 1937, *Biochem. J.* **31**: 966–973.

15. Hughes, Thomas P. 1932, *J. Bact.* **23**: 437–447.

16. Porter, J. R., and Pelczar, Michael J., Jr. 1940, *Sci.* **91**: 576–577.

17. Sevag, M. G., and Green, Morris N. 1944, *J. Biol. Chem.* **154**: 719–720.

18. Fildes, Paul, Richardson, G. M., Knight, B. C. J. G., and Gladstone, G. P. 1936, *Brit. J. Exper. Path.* **17**: 481–484.

19. Gladstone, G. P. 1937, *Brit. J. Exper. Path.* **18**: 322–333.

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21. Gladstone, G. P. 1938, *Brit. J. Exper. Path.* **19**: 208–226.

22. Parker, Julia T., Hopkins, J. G., and Gunther, Anne. 1926, *Proc. Soc. Exper. Biol. and Med.* **23**: 344–346.

23. Bigger, Joseph W. 1933, *J. Path. Bact.* **36**: 87–114.

24. Casman, E. P. 1940, *J. Bact.* **39**: 84.

25. Duthie, E. S., and Wylie, J. A. H. 1945, *Brit. J. Exper. Path.* **26**: 130–136.

influence on the production of various soluble bacterial toxins.

The present study was devoted mainly to the investigation of enterotoxin formation in progressively simplified mediums, beginning with a medium similar to one used by Gladstone,²¹ which contained 16 amino acids. In addition attention has been given to the effect of glucose on enterotoxin formation in simple mediums and to quantitative assay methods.

MATERIALS AND METHODS

Cultures.—Six strains of *Staphylococcus aureus* were used in these experiments. The following is a brief history and description of characteristics of each strain:

Strain 8 was isolated in 1930 by Dr. G. M. Dack from a cake causing several cases of food poisoning in Milwaukee. It produces enterotoxin and alpha-lysin but no beta-lysin production has been noted.

Strain 147 was received in this laboratory in 1933 from Dr. Litterer, Tennessee State Department of Health. It was isolated from food involved in an outbreak of food poisoning. It also produces enterotoxin and alpha-lysin but no detectable beta-lysin.

Strain 161 was isolated in 1935 by Dack, Bowman and Harger from sandwiches causing a food poisoning outbreak at Indianapolis. This strain has been consistently enterotoxigenic in this laboratory since the time of isolation. It produces alpha-lysin; beta-lysin production has been observed on blood agar plates but not by titration of culture supernatants.

Strain 196 was isolated in 1940 by Dr. G. G. Slocum from ham involved in a food poisoning outbreak and was received in this laboratory in 1940. It produces enterotoxin and both alpha- and beta-lysins.

Strain 184 was isolated in 1939 by Dr. G. G. Slocum from ham involved in a food poisoning outbreak. It was received in this laboratory in 1940. This strain produces both alpha- and beta-lysins but numerous tests for enterotoxin production in this laboratory have been negative.

Strain J-32-A was received at the University in 1944 from Dr. C. E. Dol-

TABLE 1.—Composition of medium 1.

Component	Concentration	
	Molarity	Grams per liter
<i>l</i> (+)-arginine HCl	M/110	1.909
<i>l</i> (-)-cystine	M/10,000	0.024
<i>dl</i> -alanine	M/1,500	0.059
glycine	M/198	0.378
<i>dl</i> -phenylalanine	M/4,000	0.041
<i>dl</i> -valine	M/1,500	0.078
<i>l</i> (-)-aspartic acid	M/1,500	0.089
<i>l</i> (+)-glutamic acid	M/1,500	0.098
<i>l</i> (-)-histidine 2HCl	M/4,000	0.057
<i>l</i> (-)-hydroxyproline	M/1,500	0.087
<i>l</i> (-)-leucine	M/1,500	0.087
<i>l</i> (+)-lysine 2HCl	M/4,000	0.055
<i>dl</i> -methionine	M/4,000	0.037
<i>l</i> (-)-tryptophane	M/20,000	0.010
<i>l</i> (-)-tyrosine	M/4,000	0.045
<i>l</i> (-)-proline	M/280	0.411
nicotinic acid	M/100,000	0.001,23
thiamine hydrochloride	M/10,000,000	0.000,033,4
glucose (anhydrous)	M/80	2.25
MgSO ₄	M/6,000	0.020
FeSO ₄ ·(NH ₄) ₂ SO ₄ ·6H ₂ O	M/20,000	0.0145
KH ₂ PO ₄	M/30	4.536
Redistilled water		

man. This strain produces beta-lysin but production of alpha-lysin has not been detected and numerous tests for enterotoxin have been negative.

The hemolytic characteristics of these strains were determined in a previous investigation by the observation of plate hemolysis and by detection of soluble hemolysins in cultures.²⁶ The data concerning enterotoxin production is derived from the same source and from several investigations by other workers in this laboratory.

Mediums.—Experimental mediums were made from commercially prepared amino acids, vitamins, glucose and inorganic salts. Reagent grade inorganic salts and glucose were used.

26. Surgalla, M. J., and Hite, K. Eileen. 1945, J. Infect. Dis. 76: 78-82.

The composition of the initial medium is given in table 1. Mediums were usually prepared in one liter lots and occasionally in smaller amounts. Freshly redistilled water was used at all times. Stock solutions of thiamine, nicotinic acid and pantothenic acid (used in most of the later experiments) were prepared and stored in sealed tubes in the refrigerator after sterilization by filtration through fritted glass filters (grade U F). Cystine and tyrosine solutions (prepared in N/10 HCl) were filtered and stored in the same manner. In the preparation of mediums measured portions of these solutions were added to give the desired concentrations. Other components were weighed in the dry state each time a lot of medium was prepared. During most of the study all components of the medium were combined, the pH was adjusted to 7.6 with N/1 NaOH, and the medium was dispensed into the appropriate culture vessels and sterilized in the autoclave. The first few mediums prepared were sterilized at 10 lbs. for 10 minutes; however, because of contamination in one lot, mediums were subsequently sterilized for 20 minutes.

Growth experiments.—Test tube experiments were carried out with most of the mediums to determine their relative suitability for growth of the staphylococcus strains. In comparing 2 mediums, a series of tubes, each containing 10 ml of medium, was inoculated with 0.1 ml amounts of 10-fold dilutions (in phosphate buffer, pH 7.6) of culture. Cultures for inoculation were grown in synthetic medium (usually the simpler of the mediums being tested). A control series of tubes of veal infusion broth or the complete synthetic medium was inoculated at the same time. Growth (as determined by inspection) was recorded at half-day intervals for several days. At first, turbidity was recorded as

+ (slight), ++ (moderate), +++ (heavy) and ++++ (maximum). This admittedly crude and subjective method was satisfactory for comparison of different mediums in a single experiment. Later, in order to compare results of different experiments on a more objective basis, growth was determined by comparing turbidity of cultures with McFarland nephelometer standards, and records were made in terms of numbers corresponding to the number of the nearest matching standard tube.

By this type of growth experiment, rate and quantity of growth resulting from a series of graded inoculums could be compared in different mediums. It was possible to demonstrate that in all of the mediums which supported growth very small inoculums were sufficient to initiate growth. In most experiments inoculums of 10^{-7} to 10^{-9} ml of culture initiated growth in the synthetic as well as in the veal infusion medium.

In the last part of the study growth was determined by examining the turbidity of flask cultures at the end of the incubation period. Growth was much more abundant in rotating flasks under increased CO_2 tension (vide infra) than in stationary test tubes without additional CO_2 . Furthermore, considerable differences in growth of strains in extremely simple mediums were sometimes noted in flask cultures which were not apparent in test tube experiments.

Methods for production of enterotoxin.—Culture mediums were inoculated with 0.1 ml of a 1:1000 dilution (in phosphate buffer solution, pH 7.6) of an 18–48 hour culture in synthetic medium, usually the test medium. When growth was slow in the test medium older cultures were occasionally used for inoculation.

In the early part of the study cultures for production of enterotoxin were incubated in 35–50 ml quantities in Pyrex

nursing bottles; later, 500 ml capacity Florence flasks containing 85–100 ml of culture medium were used. After inoculation, 20% CO₂ was introduced manometrically through cotton plugged glass tubing extending through the rubber stoppers. The glass tubes were then sealed in a flame and the flasks were incubated for 3–7 days at 37 C. During incubation the flasks were kept in continuous motion on a rotating wheel. The speed of rotation of the wheel ranged from 30 to 330 revolutions per hour.

After incubation, cultures were centrifuged and the supernatants were tested for enterotoxin and hemolysins.

Assay of enterotoxin.—Two methods were used to detect the presence of enterotoxin in staphylococcus cultures. In the first, supernatants were fed to young rhesus monkeys (*Macaca mulatta*) by stomach tube.²⁷ Thirty-six monkeys were used in these studies. Each was found to be susceptible when fed culture supernatants of enterotoxigenic strains prior to use. Tests for continued susceptibility were made in the same way at intervals during the study and negative reactors were eliminated from the experiments. In the second method, supernatants heated for 30–35 minutes in boiling water were injected intravenously into cats.²⁸ Cats weighing from 520 to 3,960 grams were used throughout the experiments, each one seldom being injected more than 3 times. Only cats which appeared to be in good health were used, since vomiting is one of the symptoms of panleucopenia

a very common and highly infectious type of feline epizootic.^{29,30,31,32} There was no indication in this study that spontaneous vomiting occurs in healthy animals. Monkeys were observed for 5 hours after feeding and cats for 3 hours after injection; vomiting was considered to be a positive reaction for enterotoxin.

Assay of hemolysins.—Supernatants were titrated for lytic activity against rabbit and sheep erythrocytes. Two-fold dilutions of toxin were made in saline, and 0.5 ml of 2% suspension of red cells (washed 3 times) was added to 0.5 ml of toxin in Wasserman tubes. The tubes were shaken and placed in a 37 C water bath; readings were made after one hour. The minimum hemolytic dose (M.H.D.) of alpha-hemolysin as defined by Dolman³³ is "the volume of toxin which just suffices to hemolyse completely one cc of a 1% suspension in normal saline of rabbit erythrocytes after one hour's incubation at 37 C." In titrating beta-hemolysins the rabbit red cells were replaced by sheep cells and final readings were made after incubation at 37 C for one hour followed by cooling for one hour in an ice-water bath. Since the toxins were titrated in half milliliter amounts the M.H.D. in each case would be twice the reciprocal of the greatest dilution causing complete hemolysis.

Alpha-hemolytic supernatants (e.g. strains 8 and 147) which hemolyse rabbit red blood cells also lyse sheep cells in somewhat lower dilutions. Supernatants of pure beta-hemolytic strains, such as J-32-A, produce "hot-

27. Jordan, E. O., and McBroom, Josephine. 1931, *Proc. Soc. Exper. Biol. and Med.* **29**: 161–162.
28. Davison, Ellen, Dack, G. M., and Cary, W. E. 1938, *J. Infect. Dis.* **62**: 219–223.
29. Hammon, W. D., and Enders, J. F. 1939, *J. Exper. Med.* **69**: 327.
30. Hammon, W. D., and Enders, J. F. 1939, *J. Exper. Med.* **70**: 557.

31. Lawrence, J. S., and Syverton, J. T. 1938, *Proc. Soc. Exper. Biol. and Med.* **38**: 914–918.
32. Lawrence, J. S., Syverton, J. T., Shaw, J. S., Jr., and Smith, F. P. 1940, *Am. J. Path.* **16**: 333.
33. Dolman, C. E., and Kitching, J. S. 1935, *J. Path. Bact.* **41**: 137–162.

cold" lysis of sheep red cells but little or no lysis of rabbit cells. Therefore, the methods used in this study will suffice to detect both alpha- and beta-lysins in a culture containing a mixture of these lysins only if the beta-titer is as high or higher than that of the alpha-lysin, as in cultures of strains 184 and 196. Other methods are necessary to demonstrate beta-lysin production by a strain, such as 161, which produces very little beta- as compared with alpha-lysin. Production of beta-lysin by strain 161 has been

and Hite.²⁶ Other methods which may be used for detecting beta-lysin in a mixture are inactivation of alpha-lysin by (1) heating at 55–60 C for 30–60 minutes, (2) neutralization with anti-toxin, or (3) differential formaliniza- tion.^{37,38,35,39}

Growth and toxin production in relatively complex synthetic mediums

Three strains of staphylococci (196, 161 and 147) were grown in each of 3

TABLE 2.—*Growth and production of hemolysins and enterotoxin by three strains of staphylo- cocci in three experimental mediums.*

Medium	Strain	Enterotoxin						Hemolysins		Growth*
		Monkey feeding tests			Cat intravenous tests			Rabbit erythrocytes minimum hemolytic dose	Sheep erythrocytes minimum hemolytic dose	Days required for apparent growth in test tube cultures
		Dose (ml)	Reaction		Dose (ml)	Reaction				
			+	—		+	—			
1 †	147	50	0	2	2.0	0	1	32	8	6½
	196	45-50	1	1	2.0	1	0	64	64	3½
	161	50	2	0	2.0	0	1	64	16	3½
2 ‡	147	50	0	1				4	4	1½
	196	50	0	1				4	256	1
	161	50	0	1				4	4	1
3 §	147	50	0	1				4	4	2½
	196	50	1	0				16	256	2
	161	50	0	1				16	8	2

* In this experiment only one tube of each medium was inoculated with each strain. The inoculum for each tube was 0.1 ml of a 1:100,000 dilution of a 32 hr culture in Medium 1.
† Medium 1 contains 16 amino acids (see table 1).
‡ Casein hydrolysate medium.
§ Medium 1 minus aspartic acid, glutamic acid, histidine, hydroxyproline, leucine, lysine and tyrosine.

demonstrated by the occurrence of typical zones of "hemolysis" about colonies on 2% sheep's blood agar plates; at 37 C some colonies produce an inner zone of complete clearing (caused by alpha-lysin) surrounded by a darker incom- pletely hemolysed zone (caused by beta lysin) which becomes lighter on cooling. Plate methods for distinguish- ing alpha- and beta-lysin have been used by Naidu,³⁴ Bryce and Rountree,³⁵ Christie and Graydon³⁶ and Surgalla

different mediums in the initial experi- ments to determine whether enterotoxin could be produced in "amino acid media." The composition of Meduim 1 is given in table 1. Medium 2, a casein hydrolysate medium included as a con- trol on culture methods, was composed of 1.5% "vitamin free" casein hydroly- sate (Wyeth), 10⁻⁵M nicotinic acid, 10⁻⁷M thiamine HCl and M/80 glucose. Medium 3 contained 9 amino acids

34. Naidu, P. M. N. 1934, Zbl. Bakt. I Abt. Orig. 132: 47–57.
35. Bryce, Lucy M., and Rountree, Phyllis M. 1936, J. Path. Bact. 43: 173–189.
36. Christie, R. and Graydon, J. J. 1941, Aust. J. Exper. Biol. 19: 9–16.
37. Bigger, Joseph W., Boland, C. R., and O'Meara, R. A. Q. 1927, J. Path. Bact. 30: 271–277.
38. Glenny, A. T., and Stevens, Muriel F. 1935, J. Path. Bact. 40: 201–210.
39. Smith, M. Llewellyn and Price, S. A. 1938, J. Path. Bact. 47: 361–377.

(alanine, arginine, cystine, glycine, methionine, phenylalanine, proline, tryptophane and valine) in the concentrations indicated in table 1, and was otherwise identical with Medium 1.

The results of this experiment are summarized in table 2. Growth initiated by a 10^{-6} ml inoculum occurred most

in Medium 3 and the titers in the casein hydrolysate medium were less than 4 M.H.D. for all strains. Strain 196 produced beta-lysin titers of 256 M.H.D. in Mediums 2 and 3 and 64 M.H.D. in Medium 1. Three monkey feeding tests for the presence of enterotoxin in casein hydrolysate cultures were negative. One

TABLE 3.—Growth of strain 196 in four experimental mediums.

Medium	Inoculum* (ml)	Turbidity† after incubation at 37 C						
		Days						
		$\frac{1}{2}$	1	1½	2	3	4	5
1 (16 amino acids; thiamine autoclaved)	10^{-6}	1	3	3	4			
	10^{-6}	1	2	3	4			
	10^{-7}	0	1	3	4			
	10^{-8}	0	1	3	4			
	10^{-9}	0	0	0	0	0	0	0
1 (16 amino acids; thiamine filtered)	10^{-1}	4						
	10^{-2}	3	4					
	10^{-3}	2	4					
	10^{-4}	1	3	4				
	10^{-5}	1	3	3	4			
	10^{-6}	1	2	3	4			
	10^{-7}	0	1	3	4			
	10^{-8}	0	1	3	4			
	10^{-9}	0	0	0	0		0	0
2 (Casein hydrolysate)	10^{-1}	4						
	10^{-2}	4						
	10^{-3}	3	3	4				
	10^{-4}	2	3	4				
	10^{-5}	1	2	4				
	10^{-6}	0	2	4				
	10^{-7}	0	2	4				
	10^{-8}	0	1	4				
	10^{-9}	0	0	3	4			
3 (9 amino acids)	10^{-1}	3	3	3	4			
	10^{-2}	2	2	3	4			
	10^{-3}	0	1	2	3	4		
	10^{-4}	0	1	2	3	4		
	10^{-5}	0	0	0	1	3	4	
	10^{-6}	0	0	1	2	4		
	10^{-7}	0	0	0	0	1	2	3
	10^{-8}	0	0	0	0	1	1	3
	10^{-9}	0	0	0	0	0	0	0
Veal infusion broth	10^{-1}	4						
	10^{-2}	4						
	10^{-3}	4						
	10^{-4}	4						
	10^{-5}	4						
	10^{-6}	4						
	10^{-7}	4						
	10^{-8}	4						
	10^{-9}	0	0	0	0	0	0	0

* A 32 hr culture in Medium 1 was used.

† Turbidity determined by inspection; 1 (slight), 2 (moderate), 3 (heavy) and 4 (maximum).

quickly in the casein hydrolysate medium, and appeared sooner in Medium 3 than in Medium 1 (later experiments showed Medium 1 to be superior to Medium 3). Alpha-lysin production was best in Medium 1; titers of 64 M.H.D. were produced by strains 161 and 196, and 32 M.H.D. by strain 147. These titers were 4 to 8 times those produced

of 3 monkey feeding tests with Medium 3 cultures was positive. Three of 6 monkeys and one of 3 cats tested with Medium 1 cultures gave positive reactions.

Following this demonstration that enterotoxigenic strains of staphylococci could produce enterotoxin in mediums containing 16 and 9 amino acids, fur-

ther experiments were conducted with mediums simplified by omitting certain amino acids. In addition the effects of several other factors on growth and enterotoxin production were investigated. No further attempts were made to produce enterotoxin in casein hydrolysate media.

Sterilization of thiamine.—In the preparation of mediums for initial experiments thiamine was sterilized by filtration to avoid possible destruction by heating. In an experiment designed to determine whether thiamine could be

inoculums ranging from 10^{-1} to 10^{-10} ml of a 22 hour culture of strain 196 in Medium 1. In one series of tubes the cystine and methionine had been sterilized in the autoclave and in the other by filtration. Growth occurred in all tubes except those receiving 10^{-9} and 10^{-10} ml inoculums. No difference was found in the rate or abundance of growth in pairs of tubes receiving the same inoculum. Therefore, cystine and methionine were subsequently added to mediums before sterilization in the autoclave.

TABLE 4.—*Effect of time of incubation on hemolysin and enterotoxin production.*

Incubation time (days)	Enterotoxin				Hemolysins	
	Monkey feeding tests		Cat intravenous tests		Rabbit erythrocytes minimum hemolytic dose	Sheep erythrocytes minimum hemolytic dose
	Dose (ml)	Reaction	Dose (ml/kg)	Reaction		
3	50	0	1.0	+	64	256
	40	+	2.0	+		
5	45	0	2.0	0	128	256
	50	+	2.0	+		
7	50	0	2.0	+	64	256
	45	+	2.0	+		

In these experiments strain 196 was grown in Medium 1 with proline concentration reduced to M/690.

sterilized in the autoclave, growth of strain 196 in 2 lots of Medium 1 was compared; in one lot thiamine was sterilized in the autoclave and in the other by filtration. As shown in table 3, growth was the same in tubes receiving an identical inoculum whether thiamine had been sterilized in the autoclave or by filtration. Thiamine, therefore, was subsequently added to mediums before sterilization in the autoclave.

Sterilization of cystine and methionine.—Fildes and Richardson²⁰ reported that autoclaved cystine and methionine were inhibitory to growth and should, therefore, be sterilized by filtration. An experiment was performed to determine whether cystine and methionine are inhibitory when added to the medium before sterilization in the autoclave at 10 lbs. for 10 minutes. Two series of tubes of Medium 1 received

Effect of reducing proline concentration.—Gladstone²¹ reported that proline concentrations of M/140 to M/700 were optimum for production of alpha-lysin. A lot of Medium 1 with proline concentration reduced from M/280 to M/690 was prepared. Comparative growth tests showed that strain 196 grew equally well in both preparations. In testing this medium for production of enterotoxin it was decided to determine in the same experiment whether any detectable increase in toxin production would occur at incubation periods longer than 3 days. Nine Pyrex nursing bottles containing 45 ml of this medium were inoculated with 0.1 ml portions of a 1:10,000 dilution of a 20 hour culture of strain 196 in Medium 1. Three flasks were incubated on the wheel for 3 days, 3 flasks for 5 days and 3 flasks for 7 days. Enterotoxin was detected in all of

the cultures and the hemolysin titers (64–128 M.H.D. alpha and 256 M.H.D. beta) were comparable at all 3 incubation periods (table 4).

Effect of biotin.—Porter and Pelczar¹⁶ reported that certain strains of staphylococci could not be subcultured indefinitely in their chemically defined medium unless biotin was added. They found that biotin was active in concentrations as low as 0.0001 $\mu\text{g}/\text{ml}$. Comparative growth tests similar to those described above were made to test the effect of biotin in concentrations of 0.0005 $\mu\text{g}/\text{ml}$ on growth of strain 147 in Medium 1. A second experiment was designed to test the effect of 0.05 $\mu\text{g}/\text{ml}$ of biotin on growth of strain 196 in a "6 amino acid medium" (alanine, arginine, cystine, glycine, phenylalanine and valine), and this was repeated in a third experiment using a biotin concentration of 0.1 $\mu\text{g}/\text{ml}$. In each of these experiments there appeared to be a very slight increase in rate of growth in some of the tubes containing biotin; the differences were, however, considered to be questionable. Strain 196 was carried through 30 subcultures in Medium 1 and then through an additional 30 in several of the more simple amino acid mediums. In view of the questionable evidence that biotin has any stimulating effect on these 2 strains, it was not used subsequently.

Effect of tryptophane and pantothenic acid.—During the course of the above experiments it was noted that strain 147 seemed to grow a little less rapidly and less abundantly than the other strains.

In view of Gladstone's¹⁹ finding that 4 of 25 strains required tryptophane the growth of strain 147 was compared in Medium 1 containing this amino acid in concentrations of M/20,000 and M/4,000. No differences in rate or quantity of growth in the 2 mediums were observed.

Sevag and Green¹⁷ observed that pantothenic acid supported growth of tryptophane-deficient strains in the absence of tryptophane if glucose was present. The stimulating effect of this vitamin was tested using strain 196 in a medium devoid of tryptophane. Growth of this strain was compared in Medium 4 (containing the 7 amino acids, alanine, arginine, cystine, glycine, phenylalanine, proline and valine) with and without calcium pantothenate in a concentra-

TABLE 5.—*Effect of calcium pantothenate on growth of strain 196.*

Medium	Inoculum* (ml)	Turbidity† after incubation at 37 C				
		Days				
		$\frac{1}{2}$	1	1 $\frac{1}{2}$	2 $\frac{1}{2}$	4 $\frac{1}{2}$
4‡	10 ⁻¹	3	3	3	3	3
	10 ⁻²	2	3	3	3	3
	10 ⁻³	0	2	3	3	3
	10 ⁻⁴	0	1	2	3	3
	10 ⁻⁵	0	0	1	2	3
	10 ⁻⁶	0	0	1	2	3
	10 ⁻⁷	0	0	0	2	3
	10 ⁻⁸	0	0	0	2	3
4‡ +pantothenate 1 $\mu\text{g}/\text{ml}$	10 ⁻¹	3	3	3	4	
	10 ⁻²	2	3	3	4	
	10 ⁻³	0	2	3	4	
	10 ⁻⁴	0	1	2	4	
	10 ⁻⁵	0	0	1	3	4
	10 ⁻⁶	0	0	1	3	4
	10 ⁻⁷	0	0	0	3	4
	10 ⁻⁸	0	0	0	0	0

* An 18 hr culture in Medium 3 (9 amino acids) was used.

† Turbidity was determined by inspection; 1 (slight), 2 (moderate), 3 (heavy) and 4 (maximum).

‡ Medium 4 contained the amino acids, alanine, arginine, cystine, glycine, phenylalanine, proline and valine.

tion of 1 $\mu\text{g}/\text{ml}$. Growth was more abundant in the tubes containing pantothenate (table 5). Toxin production by strain 196 was tested in these media. Two monkey feeding tests with culture supernatants of each medium were negative; 1 of 2 cat tests with each was positive. Alpha-lysin titers were 16 and 32 M.H.D. and beta-lysin titers were 32 M.H.D. Because of the observed stimulating effect on growth by calcium pantothenate this vitamin was incorporated (1 $\mu\text{g}/\text{ml}$) in all subsequent mediums.

Simplification of mediums by elimination of amino acids

The demonstration of enterotoxin

production in Medium 4 indicated that at least 9 of the 16 amino acids in Medium 1 were unnecessary. The mediums were further simplified by the elimination of other amino acids.

Effect of proline, hydroxyproline and glutamic acid.—An experiment was performed to determine whether hydroxyproline or glutamic acid exert any effect in the absence of proline. Three lots of a medium containing the 6 amino acids, alanine, arginine, cystine, glycine, phenylalanine and valine in the usual con-

days incubation was approximately the same in all 3 mediums. There were no significant differences in hemolysin titers in supernatants of 5-day cultures in the 3 mediums. Alpha-lysin titers in duplicate experiments were 32–64 M.H.D. and beta-lysin titers were 64–256 M.H.D. Enterotoxin was produced in all 3 mediums. Four monkey tests and 3 cat tests were performed with each of the culture supernatants. Two monkeys and two cats reacted positively to the culture supernatant of the

TABLE 6.—*Production of hemolysins and enterotoxin in mediums containing two to five amino acids.*

Medium*	Strain	Enterotoxin						Hemolysins; minimum hemolytic dose	
		Monkey feeding tests			Cat intravenous tests				
		Dose (ml)	Reaction		Dose (ml/kg)	Reaction		Rabbit erythrocytes	Sheep erythrocytes
			+	—		+	—		
5	196	35–100	0	4	2.0–4.0	2	1	16	32–64
	8	40–55	0	2	2.0–4.0	0	3	16	4
	161	50	0	1	2.0	1	2	16	8
6	196	40–100	0	2	1.4–2.0	2	0	8	16
	161	60	0	1	3.0	2	0	8	8
7	196	40–100	0	3	2.0	1	1	8–16	16–64
	8	45–50	0	2	2.0–3.0	1	1	8–16	4–8
	161	50	0	1	2.0	1	1	4	8
8	196	60–150	0	2	2.0	1	0	4	16
	161	45	0	1	2.0	1	1	4	8
9	196	40–100	0	2	2.0	2	0	8	16
	161	60	0	1	2.0–3.0	1	1	8	4
10	196	55–100	0	2	2.0	1	1	8	16
	161	50	0	1	2.0	0	2	4	4
11	196	55–100	0	2	2.0	0	2	4	8
	161	60	0	1	2.0	0	2	4	4
12	161	45–70	2	5	1.0–3.0	6	7	4–16	8

* The mediums contain the following sources of nitrogen:
5—cystine, arginine, glycine, phenylalanine and valine.
6—cystine, arginine, glycine, phenylalanine and alanine.
7—cystine, arginine, glycine and phenylalanine.
8—cystine and twice the usual concentrations of arginine, glycine and phenylalanine.
9—cystine, arginine and glycine.
10—cystine, arginine and ammonium sulfate (M/250).
11—cystine and arginine.
12—cystine, arginine (M/50), glycine (M/100) and ammonium sulfate (M/250).

centrations were prepared. To one of these was added hydroxyproline (M/280) and to another glutamic acid (M/280). Strain 196 was tested in these 3 mediums for growth and toxin production. Hydroxyproline had no effect on growth. Glutamic acid seemed to retard growth; however, it was noted that the final amount of growth after 4

medium containing only 6 amino acids; one monkey and two cats reacted positively to each of the others.

Effect of alanine, glycine, phenylalanine and valine.—In the next series of experiments the amino acids alanine, glycine, phenylalanine and valine were found to be unnecessary for production of enterotoxin (table 6). Turbidity of

test tube cultures corresponded to McFarland numbers 1 to 2 in Mediums 5 and 6 (containing 5 amino acids) and in Mediums 7 and 8 (4 amino acids). Although 3 of the amino acids in Medium 8 were present in double the usual concentration, no beneficial effect was noted. Growth was less abundant in Medium 9 which contained 3 amino acids, and in Medium 10 which contained 2 amino acids and ammonium sulfate. Growth was poorest in Medium 11 which contained only cystine and arginine. Very good growth occurred in Medium 12; turbidity readings corresponding to McFarland numbers 4 to 5 were obtained in flask cultures. This medium contained the usual concentration of cystine, approximately twice the usual concentrations of arginine and glycine, and M/250 ammonium sulfate.

Hemolysin production in these mediums was, in general, poor. The highest titers (16 M.H.D. alpha- and 64 M.H.D. beta-lysin) were produced in Mediums 5 and 7, and lysins were barely detectable in Medium 11.

Enterotoxin was detected by the cat test in supernatants of cultures in all of these mediums with the exception of Medium 11. Only supernatants of cultures in Medium 12 provoked vomiting in monkeys.

In an experiment not recorded in table 6, a medium whose only source of nitrogen was cystine (M/10,000) and ammonium sulfate (M/250) was inoculated with strain 196. Growth was very poor in 7-day cultures. Two monkeys fed 65 and 70 ml of culture supernatant did not vomit. Eight cats, injected in duplicate with doses of 0.5, 1.0, 2.0 and 3.0 ml/kg also failed to react.

Effect of glucose concentration.—A concentration of 0.225% glucose (anhydrous) has been used in the experimental mediums described thus far. The terminal p_H of supernatants of cultures grown

in these mediums has ranged between 6.0 and 7.0. Since glucose concentration has been found to influence production of other bacterial toxins, experiments were performed to determine the effect on growth and toxin production of glucose concentrations of 0, 1, 10 and 20%. All of these experiments were performed in Medium 12, which contained the 3 amino acids, cystine, arginine and glycine.

There was no visible growth in cultures in mediums containing no glucose, and pour plates inoculated with 4-day cultures remained sterile. Three cats injected with boiled culture supernatants in doses of 2 to 3 ml/kg did not vomit.

Abundant growth occurred in mediums containing 1.0% glucose, becoming grossly apparent in flask cultures after 1.5 days incubation. In 3 experiments turbidity of 4-5 day cultures corresponded to McFarland numbers 5 to 8.5, and the p_H of culture supernatants was 4.7 to 4.8. One of 12 monkey tests and two of 6 cat tests with supernatants of these cultures were positive.

Growth in mediums containing 10% glucose was also abundant, but turbidity did not develop in flasks until after 2.5 days incubation. Turbidity of these cultures corresponded to McFarland numbers 4.5 to 8, and the p_H of supernatants was 4.9 to 5.1. One of 9 monkeys and two of 5 cats reacted positively to these culture supernatants.

Growth was delayed in the mediums containing 20% glucose. There was no gross evidence of growth in 4-5 day cultures, although plate counts indicated that growth had occurred, and the p_H of supernatants was 6.8 to 6.9. Five monkeys and 4 cats reacted negatively to supernatants of 4-5 day cultures. One culture incubated for 9 days became turbid on the sixth day. Turbidity after 9 days corresponded to McFarland

number 2 and the p_H of the supernatant was 6.0. One monkey fed 45 ml of supernatant reacted negatively and one cat injected with a dose of 3 ml/kg body weight of boiled supernatant vomited.

In 2 instances the p_H of culture supernatants was raised from 4.8–5.0 to 7.1–7.2 before boiling in order to investigate the possibility that boiling at the low p_H might decrease the potency of enterotoxin more rapidly than boiling

for 30 minutes or more at boiling temperature. Two cats injected with preparations containing beta-lysin vomited; one of these received a dose of 0.5 ml/kg of beta-hemolytic filtrate heated for only 10 minutes in boiling water and the other received 1.0 ml/kg of an unheated beta-hemolytic filtrate; the latter animal died 2 days later. No reaction occurred in one cat receiving 0.08 ml/kg and one cat receiving 1.0 ml/kg of the

TABLE 7.—*Titration of enterotoxin by the intravenous cat method.*

Experiment Number	Medium	Strain	Enterotoxin (ml culture supernatant/kg body wt)					
			0.025	0.05	0.1	0.25	0.5	1.0
1	1 M/620 proline	196			1/4*	4/7	3/4	2/4
2	1 no proline	196 147		0/2	0/4 0/4		0/2 1/4	1/2 4/4
3	1	196	0/2	0/2	2/2			
	1 no proline	196	0/2	0/4	0/2	2/4	2/2	
	1 no arginine	196	0/2	0/2	0/2	0/4	1/2	
4	a	161	0/2	0/2	0/2	0/2	2/2	2/5
	12 b			0/2	0/2	0/3	0/2	0/2

* Numerator indicates the number of positive reactions. Denominator indicates the number of animals inoculated.

at neutrality. One of the preparations (10% glucose culture) provoked vomiting in a cat and the other (1% glucose culture) did not (these results are included among those recorded above). The investigation was not further continued.

Negative control cat tests.—Woolpert and Dack³ have called attention to the necessity of negative controls in parenteral tests for enterotoxin. These should include consistently negative results with non-enterotoxigenic strains as well as with uninoculated mediums.

Twenty-three previously unused cats were injected with uninoculated culture mediums or with culture supernatants or filtrates of nonenterotoxigenic strains. No vomiting reactions occurred in any of 18 cats receiving 1–2 ml/kg body weight of uninoculated mediums or culture supernatants which were heated

sample heated for 10 minutes; one cat received 1.0 ml/kg of the unboiled preparation without apparent effect. The beta-lysin titers of these 2 filtrates were 256 and 2,048 M.H.D./ml respectively. Many additional negative control tests were performed with cats which had been used previously. These were all negative. However, the cats may have been immunized to beta-lysin by the previous injections. These observations, similar to those of other workers,^{40,41} indicate that non-specific vomiting caused by medium components or by hemolysins in adequately heated culture supernatants is not an important source of error in the intravenous cat test for enterotoxin.

40. Hammon, William McD. 1941, Am. J. Pub. Health 31: 1191–1198.

41. Dolman, C. E. 1943, Canad. J. Pub. Health 34: 97–111 and 205–235.

*Experiments on quantitative
assay of enterotoxin*

The methods for detection of enterotoxin used thus far have merely indicated its presence or absence in culture supernatants. An attempt was made, however, to develop a quantitative cat test for assay of enterotoxin. The results of these experiments are summarized in table 7.

In the first experiment 19 injections were made with doses of 0.1, 0.25, 0.5 or 1.0 ml/kg of enterotoxic preparation. Ten vomiting reactions occurred. There was no correlation between size of dose and positive reactions; however, the difference between the smallest and largest dose was only 10-fold.

In experiment 2 the dosage range was extended to include doses too small to cause vomiting. Supernatants of 5-day cultures of strains 196 and 147 in Medium 1 (proline omitted) were used. In each case the experiment was carried out on two different days, with 3 days intervening. In tests with strain 196, reactions were negative in cats receiving doses of 0.5 ml/kg or less. A dosage of 1.0 ml/kg caused one of two cats to vomit and a dosage of 2.0 ml/kg caused one of two cats to vomit. In the tests with strain 147, 4 cats receiving 0.1 ml/kg did not vomit and one of 4 cats receiving 0.5 ml/kg vomited. A dosage of 1.0 ml/kg caused all of 4 cats to vomit and a dosage of 2.0 ml/kg caused 3 of 4 cats to vomit.

In experiment 3 a new lot of Medium 1 was prepared, as was a similar medium without proline and one without arginine. Supernatants of 3-day cultures of strain 196 in these mediums were tested. The smallest doses per kg. which caused vomiting were 0.1 ml for the complete medium, 0.25 ml for the medium without proline, and 0.5 ml for the medium without arginine. The hemolysin titers in these 3 cultures were 32, 16

and 8 M.H.D. alpha-lysin and 128, 128 and 16 M.H.D. beta-lysin respectively. Turbidity of the flask cultures corresponded to McFarland numbers 5, 4.5 and 2.5 respectively, while turbidity of 3-day test tube cultures corresponded to numbers 4, 4 and 0.5. It may be noted here that the main source of nitrogen in Medium 1 is arginine and the greatly decreased growth in its absence is to be expected.

Experiment 4 in table 7 presents the results of titrations of cultures in 2 different lots of Medium 12 (see table 6). The smallest doses which provoked vomiting were 0.5 ml/kg for the first lot and 2.0 ml/kg for the second, a 4-fold difference.

It is apparent from these tests that considerable variation in susceptibility to enterotoxin exists among cats, and sharp end points indicating minimal reacting doses were not achieved. It is also apparent from the data presented in table 7 that variable results were obtained with different lots of the same medium. For these reasons the apparent differences in the enterotoxic potency of different cultures may not be attributed solely to toxin content of the preparations. It is, however, interesting to note that in the experiments using Medium 1 with and without proline, a somewhat smaller amount of toxin from cultures grown in the complete medium was required to cause a positive reaction in cats. Also, in experiment 3, the amounts of toxin necessary to cause vomiting varied inversely with the amount of growth observed in the cultures. Further work on quantitative assay methods for enterotoxin is needed.

Development of tolerance by cats.—Hammon⁴⁰ found that tolerance develops in cats after repeated inoculation, and that great individual differences occur in the apparent degree of tolerance acquired and its rapidity of devel-

opment. He states that a cat may be used 3 or 4 times before it develops too great a tolerance, but recommends that negative results should be confirmed by inoculation of at least two previously unused animals. Dolman⁴² also reported that cats given repeated intravenous injections of enterotoxin were eventu-

TABLE 8.—*Development of resistance to enterotoxin by cats.*

Cat	Dose (ml/kg)	Reaction	Day
68	0.025	+	1
	0.025	+	10
	0.025	0	14
	0.1	0	18
	0.25	0	22
72	0.025	0	1
	0.025	0	10
	0.05	0	14
	0.1	0	18
	0.25	0	22
75	0.05	+	1
	0.05	+	10
	0.05	0	14
	0.1	+	18
	0.1	0	22
61	0.1	+	1
	0.05	+	10
	0.05	0	14
	0.1	0	18
	0.25	+	22
55	0.1	+	1
	0.05	0	10
	0.1	+	14
	0.1	+	18
	0.1	0	22

A semi-solid veal infusion agar culture of strain 196 was used. Culture supernatant was heated in boiling water for 30 minutes before use.

ally able to withstand several times their initial minimal reacting doses. Since rapid development of resistance would prevent the repeated use of cats in quantitative titrations an experiment was conducted to determine the rapidity of development of resistance by cats.

Enterotoxin produced by growing strain 196 in veal infusion agar was used. A portion of this preparation was heated in boiling water for 30 minutes and was used for intravenous injection of cats. Five cats were used; each received 5 injections. The dosage was increased

after negative reactions. The results are shown in table 8. The smallest dose used, 0.025 ml/kg, provoked vomiting on two occasions in cat 68. This animal rapidly became resistant. No positive reactions occurred with cat 72. Cats 75, 61 and 55 became resistant slowly. These results indicate that considerable variation may exist in initial susceptibility of cats and in their ability to develop a resistance or tolerance to the toxin. It seems improbable, however, that the results of the titration experiments with experimental mediums were affected to any great degree by the development of resistance, since each of the titrations was completed within 3 to 6 days.

DISCUSSION

Production of enterotoxin by staphylococci has been demonstrated previously in hydrolysed protein mediums. In this study it has been shown that enterotoxin may be produced in simple mediums consisting of 2 to 16 amino acids, vitamins, inorganic salts and glucose.

Since it is assumed that those amino acids not required for growth can be synthesized by the microorganism, it would seem reasonable to expect that the amino acid requirement for toxin production should not differ from the requirement for growth. The results of this study indicate that such is the case. Growth and hemolysin production appeared to be reduced as the original "16 amino acid" medium was progressively simplified by the elimination of amino acids. The nature of the assay methods used for detection of enterotoxin is such that small differences in potency of different preparations would probably not be apparent. However, the results of monkey feeding tests and the few quantitative titrations which have been carried out in cats seem to

42. Dolman, C. E. 1944, *Canad. J. Pub. Health* 35: 337-351.

indicate that enterotoxin was also diminished as mediums were simplified. When the nitrogen content of a very simple medium was increased by addition of ammonium sulfate and by increasing the concentration of the amino acids present, good growth occurred and potent enterotoxin was produced, as evidenced by positive monkey and cat tests. When titrated in cats, however, larger quantities of this toxin were required for positive reactions than were needed when toxin from the complete medium was used.

Glucose concentration has been found to influence production of other bacterial toxins. Some workers have attributed an inhibiting effect of high glucose concentrations to the resulting drop in p_H of the medium. In these studies it was found that growth did not occur in the absence of glucose, and was delayed by high concentrations of this sugar. The p_H of cultures in buffered mediums containing 1% or 10% of glucose dropped to 5.0 or less. Enterotoxin was detected by both monkey and cat tests in cultures grown in mediums containing glucose concentrations of 0.225, 1.0 and 10.0%. One positive cat reaction also occurred with supernatant of culture grown in a medium containing 20% glucose.

Several experiments were performed on cats in an attempt to develop a quantitative method for assay of enterotoxin. The values of titrations for enterotoxin with different mediums are consistent

with the observed differences in growth in these mediums. However, the great variation in the susceptibility of animals constitutes an important variable which it has not been possible to eliminate.

SUMMARY

A study has been made of the production of staphylococcal enterotoxin in synthetic mediums consisting of amino acids, vitamins, inorganic salts and glucose. Enterotoxin has been detected in the supernatants of cultures grown in mediums containing from 2 to 16 amino acids as the source of organic nitrogen. The most simple medium used successfully contained the amino acids arginine and cystine. Evidence was not obtained that amino acids unessential for growth were necessary for enterotoxin production; both toxin production and growth seemed to be reduced in the more simple mediums, an observation attributable to the amount of available nitrogen in the mediums.

Growth did not occur in the absence of glucose in a medium containing only 3 amino acids. Enterotoxin was demonstrated in mediums containing glucose in concentrations ranging from 0.2 to 20.0%. The ability of other carbohydrates to serve as sources of energy was not investigated.

The results of experiments on titration of enterotoxin by injection of cats also suggest a reduced concentration of enterotoxin in relatively simple as compared to more complex mediums.

